# Kinin-dependent hypersensitivity reactions in hemodialysis: Metabolic and genetic factors

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**Although the association of angiotensin I-converting enzyme inhibitors (ACEis) with a negatively charged membrane is thought to be responsible for hypersensitivity reactions (HSRs) during hemodialysis, we hypothesize that these complications are due to changes in plasma aminopeptidase P (APP) activity and genotype. To test this hypothesis, we measured plasma APP activity in 14 patients who suffered HSR (HSR+) while dialyzed with an AN69 membrane and simultaneously treated with an ACEi. APP activity was also studied in a control group ($n = 39$) dialyzed under the same conditions, but who did not suffer any side effect (HSR−). We found significantly decreased plasma APP activity ($P = 0.013$) in HSR+ subjects as well as altered degradation of endogenous des-Arginine$^9$-bradykinin, with a significantly lower $\beta$ value ($P < 0.001$). The same analytical approach was taken in 171 relatives of HSR+ patients. Variance component analysis suggested that genetic differences may explain 61% of the phenotypic variability of plasma APP activity ($P < 0.001$) and the kinetic parameters that characterized kinin degradation. We also showed that the C-2399A single-nucleotide polymorphism at the *XPNPEP2* locus was a significant predictor of APP activity in the 39 HSR− controls ($P = 0.029$). Furthermore, a recessive genetic model for the A allele disclosed a significant difference in mean APP activity by genotype ($P < 0.001$). Finally, our study defined the nonspecific inhibition of recombinant APP by some ACEis. In conclusion, this paper highlights the complexity of HSR in hemodialysis, suggesting, as with angioedema, that these rare, but life-threatening adverse events are governed by several metabolic and genetic factors.**

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Although beneficial in the treatment of various cardiovascular disorders and the progression of renal failure in diabetic and non-diabetic patients,[1,2] angiotensin I-converting enzyme inhibitors (ACEis) are associated with several side effects, for which symptoms largely depend on the clinical context. Hypersensitivity reactions (HSRs) or anaphylactoid reactions, which mimic symptoms of immediate hypersensitivity, are observed in patients chronically hemodialyzed with synthetic, highly permeable, electronegative membranes while taking ACEi.[3] These reactions typically occur in the first minutes of the dialysis session, and their intensity is usually mild to moderate, but may be life-threatening. Numerous factors have been hypothesized to cause HSR.[4–7] However, reported increases in plasma bradykinin (BK) during episodes of HSR[8–10] in hemodialyzed patients suggest a role of kinins in the genesis of these adverse reactions.

The nonapeptide BK is released from high molecular weight kininogen by plasma kallikrein during activation of the contact system. It is subsequently metabolized, mainly by three zinc metallopeptidases.[11,12] ACE and X-Pro-aminopeptidase (aminopeptidase P (APP)) are the first and second inactivating metallopeptidases of BK, respectively. Carboxypeptidase *N* transforms BK into its carboxytruncated active metabolite, des-Arginine$^9$-BK (des-Arg$^9$-BK), which constitutes minor metabolic pathway unless ACE is inhibited. Des-Arg$^9$-BK is also degraded by ACE and APP. In this case, however, APP represents the main peptidase activity whereas ACE plays a secondary role.

BK exerts its pharmacological activities through its constitutively expressed kinin $B_2$ receptor,[13] although des-Arg$^9$-BK binds to the $B_1$ receptor, the synthesis of which is increased by different cytokines.[14] Both $B_1$ and $B_2$ receptor agonists have several physiological activities[13] including the ability to dilate the peripheral vasculature, directly and indirectly (resulting in hypotension), constrict

the pulmonary airways (evoking bronchospasms), and induce the release of histamine from mast cells, which are hallmarks of HSR.

Human APP, a hydrolase that cleaves N-terminal imido bonds normally protected from attack by other known aminopeptidases, exists in two known forms: a glycosylphosphatidylinositol-anchored membrane form (hmAPP) and a cytosolic form (hcAPP). mRNA of both APP forms have been found in different tissues.[15] We previously reported significantly lower plasma APP activity in patients who suffered ACEi-linked acute side effects.[16–20] When ACE was inhibited, the low APP activity was associated with an increased half-life of exogenously added[16] or endogenously generated des-Arg$^9$-BK.[19,20]

We reported recently[21] that genetic factors partially regulated the activity of APP in eight families, each with one member who developed ACEi-associated angioedema (AE). Heritability of plasma APP activity was estimated to be 34% ($\pm$25%). Sequencing the upstream region of the *XPNPEP2* gene coding for hmAPP, we found a single-nucleotide polymorphism (SNP) C-2399A, for which the A allele was linked with low plasma APP activity. In addition, we observed a deletion of 175 bp in one case of AE. This deletion resulted in a truncated protein of 38 amino acids.[21]

In the present study, we tested the hypothesis that HSR may occur not only from the association of a negatively charged membrane (physicochemical factor) and an ACEi (pharmacological agent), but is also dependent on the ability (metabolic aspect) of the dialyzed patient to degrade kinins, generated from activation of the contact system of plasma. We measured APP activity, and defined the metabolism of the endogenous kinins BK and des-Arg$^9$-BK in the plasma of 14 patients who suffered HSR (HSR+) while dialyzed with an AN69 membrane and simultaneously treated with an ACEi. These same parameters were also measured in 171 relatives of these HSR+ patients and a control group ($n = 39$) dialyzed under the same conditions, but who did not suffer any side effect (HSR−). Furthermore, we calculated the importance of heritability on APP activity, and investigated kinetic parameters among the families of HSR+ patients. Finally, we tested the nonspecific inhibition of different ACEi on a recombinant wild-type form of hmAPP.[22]

## RESULTS

### Metabolic investigations

***APP activity in plasma.*** APP values of the HSR+ patients and their relatives appear in Figure 1. Figure 2 presents the distribution of APP activity in both groups of dialyzed patients compared to that of the reference population.[12,22] Mean activity ($82 \pm 107$ U) in HSR+ plasma was significantly lower than in HSR− ($193 \pm 199$ U, $P = 0.013$) and the reference samples ($317 \pm 248$ U, $P = 0.000$). Mean plasma APP activity in the dialyzed patients corresponded respectively to the 11th (HSR+) and 38th percentile (HSR−) of the reference population. In the HSR− group, there was no significant difference in mean APP values in diabetic patients compared to other kidney diseases ($P = 0.516$). Similar results were obtained between diabetes and glomerular diseases ($P = 0.689$), diabetes, and interstitial nephritis ($P = 0.074$) (Table 1).

***Metabolism of endogenous BK and des-Arg$^9$-BK in plasma.*** Figure 3 illustrates the mean metabolic profiles of BK and des-Arg$^9$-BK during the activation of HSR+ and HSR− plasma in the presence of an ACEi. No discrepancy in BK was detected for the different kinetic parameters (Table 2), except for a lower $\alpha$ among HSR+ patients ($P = 0.043$), which characterized BK generation from high molecular weight kininogen. The main metabolic difference between HSR+ and HSR− patients occurred in des-Arg$^9$-BK degradation. In the HSR+ group, a lower $\beta$ value ($P < 0.001$) reflected the altered degradation of this $B_1$ receptor agonist by APP in the presence of an ACEi. The des-Arg$^9$-BK degradation anomaly was responsible for a higher maximal concentration of the peptide ($P = 0.048$) during the activation period. When Benjamini's correction[23] for multiple comparison was applied to these kinetic parameters, two independent of APP activity became nonsignificant ($\alpha$ for BK, $P = 0.371$; $t_{1/2}$ slope of formation ($P = 0.093$) of des-Arg$^9$-BK).

### Genetic investigations

***Heritability estimates.*** In members of the 14 families of HSR+ patients, heritability of APP was estimated at 0.61 ($\pm$0.18), indicating that genetic factors may explain 61% of the phenotypic variability in plasma APP activity ($P < 0.001$). This significant heritability estimate involved the kinetic parameters, which characterized the degradation of BK and des-Arg$^9$-BK, and depended mainly on APP activity when ACE was inhibited (Table 3). In such cases, the *P*-values obtained by Benjamini's correction did not affect the heritability estimates.

***Genotyping.*** The genotyping results of C-2399A SNP of the *XPNPEP2* gene in HSR+ and HSR− patients are presented in Table 4. The SNP was found to be a significant predictor of APP activity in the 39 HSR− controls (A and AA: $0 \pm 0$, CA: $251 \pm 167$, C and CC: $222 \pm 206$ pmol K(Dnp)PPGK(Abz) hydrolyzed/min/ml; analysis of variance: $P = 0.029$). Linear contrast on the genotyped groups was significant ($P = 0.012$). Furthermore, a recessive genetic model for the A allele disclosed a significant difference in mean APP activity by genotype (A and AA: $0 \pm 0$; CA and C and CC: $228 \pm 197$ pmol K(Dnp)PPGK(Abz) hydrolyzed/min/ml; *t*-test: $P < 0.001$). However, the SNP was not found to be predictive of HSR status as the association between groups (HSR+ and HSR−) and genotypes was not significant (Fisher's exact test: $P = 0.4289$).

### Pharmacological investigations

***Inhibition studies of recombinant APP with metallopeptidase inhibitors.*** The effect of various inhibitors on the hydrolysis of K(Dnp)PPGK(Abz)$NH_2$ by a recombinant soluble hmAPP

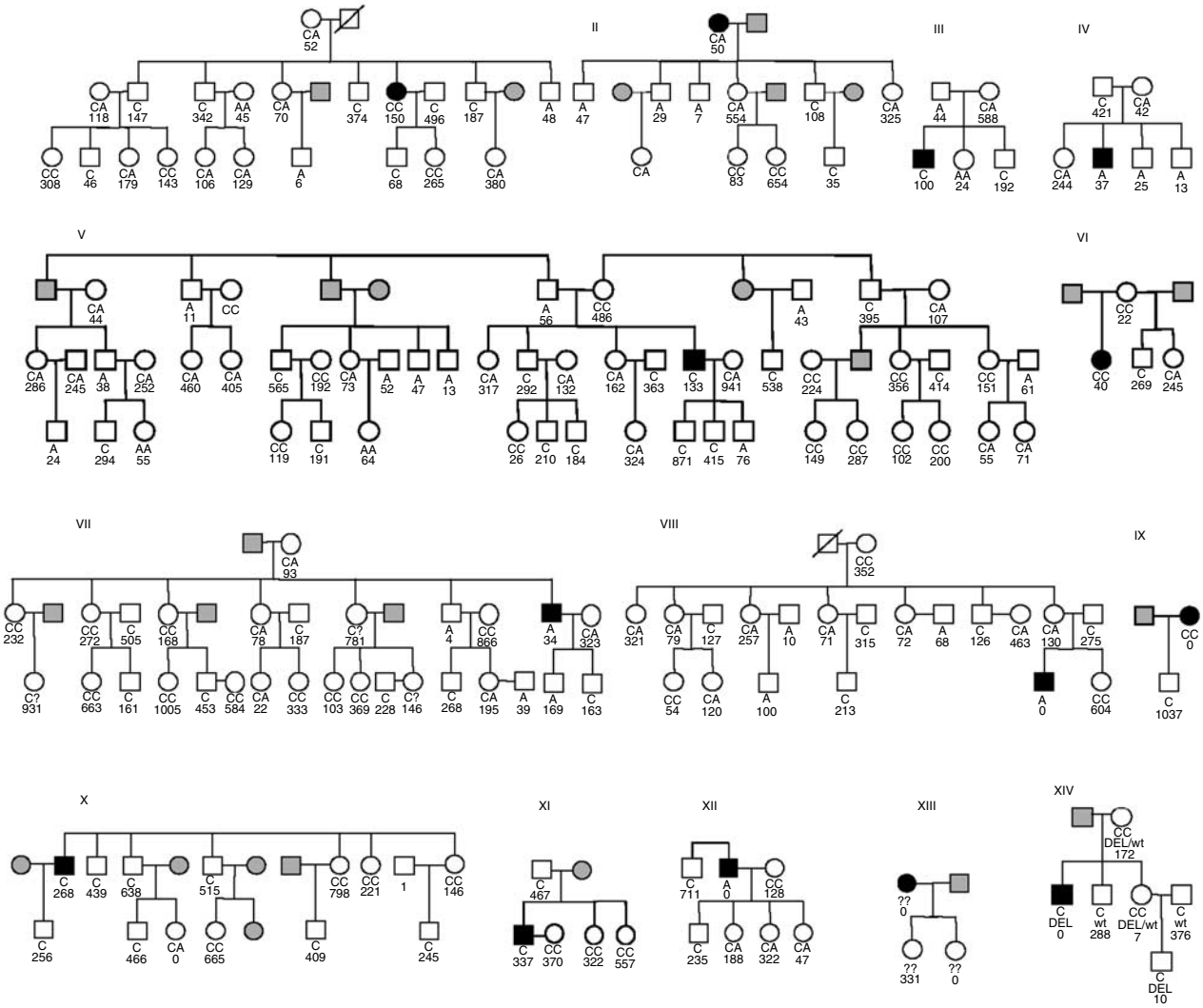


**Figure 1 | Cohorts ascertained for linkage analysis.** One member of each kindred (the propositus), depicted in black, developed HSR associated with ACEi therapy. Willing participants are depicted in white. Non-participants are shaded. The SNP genotype and the APP activity are indicated for each member of the different families. SNP genotypes were not available for family XIII.

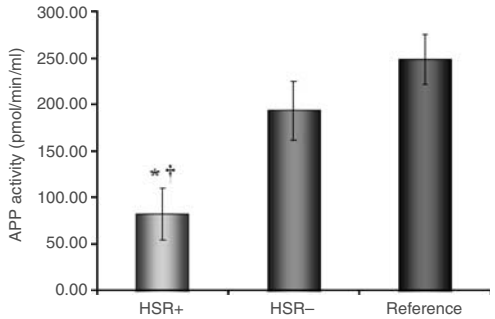


**Figure 2 | Distribution of APP activities.** Distribution of APP activities in HSR+ and HSR− patients compared with the reference population. *$P = 0.013$ HSR+ vs HSR−; †$P = 0.000$ HSR+ vs reference.

is illustrated in Figure 4. The selective APP inhibitor apstatin and the sulfhydryl group-containing captopril were the most potent inhibitors ($IC_{50}$ of $0.6 \pm 1.1$ and $17 \pm 1.1\ \mu M$, respectively). The other ACEi tested only inhibited at higher

**Table 1 | Main clinical characteristics of patients who presented HSRs during hemodialysis (HSR+) and those without (HSR−), while treated with ACEi**

| Variable | HSR+ | HSR– |
|---|---|---|
| Number | 14 | 39 |
| M/F | 5/9 | 21/18 |
| Age (extremes) | 50 (29–71) | 53 (24–78) |
| Time on dialysis (months) | 38.4±16.8 | 54.0±21.6 |
| Diabetes type 2 | 1 | 12 |
| Nephroangiosclerosis | 2 | 5 |
| Glomerular disease | 7 | 7 |
| Chronic interstitial disease | 2 | 5 |
| Polycystic disease | 1 | 5 |
| Unknown | 1 | 5 |

ACEi, angiotensin I-converting enzyme inhibitor; F, female; HSR, hypersensitivity reaction; M, male.
Values are mean±s.d.
All dialyzers were equipped with the AN69 membrane and ACEi was prescribed for more than 1 month.

concentrations (mM ranges for enalapril) or not at all (lisinopril and ramipril).

## DISCUSSION

HRS were rare in the 1980s, but this incidence increased significantly during the 1990s owing to the widespread use of ACEi in patients dialyzed with a negatively charged membrane.[24] This association prompted a warning from American federal agencies.[25]

As other rare acute side effects of ACEi, HSR could happen when at least three different factors are present (Figure 5). The first one is the presence of a drug that inhibits specifically ACE but could also nonspecifically inhibit another metallopeptidase as APP involved in the metabolism of kinins. The second factor may be the negatively charged membranes which is the physicochemical trigger for the release of kinins. The third factor is a metabolic factor which is at least in part genetically regulated. It characterizes the capacity of the dialysed patient to metabolize kinins once ACE is inhibited. In this paper, we have explored the metabolic and the genetic aspect of the AR. We have also evidenced a nonspecific inhibition of APP by some ACEi.

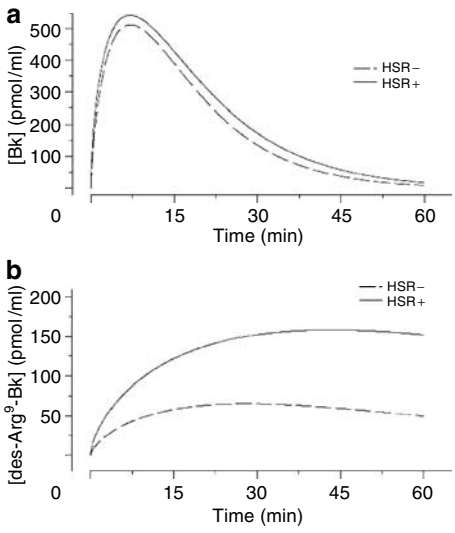


**Figure 3 | Metabolism of endogenous BK and des-Arg$^9$-BK.** Mean kinetic $\gamma$ model-fitted profiles of formation and degradation of (**a**) BK and (**b**) des-Arg$^9$-BK for HSR+ (straight line) and HSR− (dashed line) patients after activation of the contact system with glass beads in the presence of enalapril.

Despite the recent reduction of HSR incidence owing to decreased membrane surface electronegativity, there is still a persistent risk of this severe adverse reaction during hemodialysis, especially among patients taking an ACEi. As the triggering factor (Z potential of the membrane) contributing to HSR is already known, the metabolic factors predisposing to it have yet to be fully understood. In this report, we address questions concerning the factors that control the metabolism of kinins in the presence of an ACEi. In this case, the metabolism of kinins (mainly of the $\beta_1$ receptor agonist) depends on APP activity.

Although the nature of the isoform (hmAPP, hcAPP, or both) responsible for plasma APP activity remains unknown, we recently published the analysis of a genome-wide microsatellite scan, which yielded highly significant linkage between plasma APP activity and a marker flanking the *XPNPEP2* gene encoding hmAPP. Linkage was not obtained for hcAPP.[21] This suggests that APP activity in plasma is mainly or uniquely the result of hmAPP. hmAPP is mainly localized on the external site of the plasma membrane of endothelial cells, on the brush border membrane of epithelial cells in the intestine, and in renal proximal tubules.[15] hmAPP

**Table 2 | Parameters characterizing the $\gamma$ model fitted to kinetic parameters of endogenous kinins**

| | BK | | des-Arg$^9$-BK | |
|---|---|---|---|---|
| Parameter | HSR− | HSR+ | HSR− | HSR+ |
| Alpha ($\alpha$) | 0.81 ± 0.20 | 0.69 ± 0.18 | 0.87 ± 0.30 | 0.70 ± 0.16 |
| | $P$=0.043 | | $P$=0.011 | |
| Formation $t_{½}$ (min) | 1.30 ± 0.39 | 1.15 ± 0.52 | 6.54 ± 5.88 | 7.79 ± 6.83 |
| | $P$=0.247 | | $P$=0.526 | |
| Formation $t_{½}$ slope | 14 349 ± 4163 | 18 747 ± 11 041 | 451 ± 202 | 737 ± 413 |
| | $P$=0.168 | | $P$=0.031 | |
| Maximum (nM) | 523 ± 137 | 559 ± 101 | 79 ± 46 | 239 ± 275 |
| | $P$=0.375 | | $P$=0.048 | |
| Time of maximum (min) | 7.1 ± 1.4 | 7.3 ± 2.1 | 39 ± 47 | 112 ± 239 |
| | $P$=0.780 | | $P$=0.275 | |
| Beta ($\beta$) | 0.12 ± 0.04 | 0.10 ± 0.04 | 0.04 ± 0.02 | 0.02 ± 0.01 |
| | $P$=0.168 | | $P<0.001$ | |
| Degradation $t_{½}$ (min) | 21.3 ± 5.2 | 23.5 ± 6.5 | 107 ± 106 | 160 ± 124 |
| | $P$=0.218 | | $P$=0.128 | |
| Degradation $t_{½}$ slope | −2169 ± 841 | −2107 ± 1 101 | −74 ± 43 | −80 ± 58 |
| | $P$=0.829 | | $P$=0.675 | |
| AUC (pmol/ml min) | 11.8E3 ± 4.5E3 | 14.0E3 ± 4.70E3 | 16.5E3 ± 40.5E3 | 31.6E4 ± 1.04E6 |
| | $P$=0.135 | | $P$=0.300 | |

AUC, area under the curve; BK, bradykinin; des-Arg$^9$-BK, des-Argin-ine$^9$-BK; HSR, hypersensitivity reaction.
Values are means ± s.d.

**Table 3 | Heritability analysis of kinetic parameters for the kinins BK and des-Arg$^9$-BK with SOLAR**

| | BK | | | des-Arg$^9$-BK | | |
|---|---|---|---|---|---|---|
| Parameter | Heritability | Error | Probability | Heritability | Error | Probability |
| Alpha ($\alpha$) | 0.08 | 0.112 | 0.220 | 0.10 | 0.099 | 0.113 |
| Formation $t_{½}$ (min) | 0.19 | 0.136 | 0.043 | 0.29 | 0.132 | 0.004 |
| Formation $t_{½}$ slope | 0.21 | 0.108 | 0.009 | 0.37 | 0.145 | <0.001 |
| Maximum (nM) | 0.33 | 0.127 | <0.001 | 0.51 | 0.150 | <0.001 |
| Time of maximum (min) | 0.50 | 0.156 | <0.001 | 0.24 | 0.155 | 0.035 |
| Beta ($\beta$) | 0.35 | 0.138 | 0.001 | 0.36 | 0.131 | <0.001 |
| Degradation $t_{½}$ (min) | 0.56 | 0.147 | <0.001 | 0.22 | 0.153 | 0.051 |
| Degradation $t_{½}$ slope | 0.34 | 0.126 | <0.001 | 0.39 | 0.151 | <0.001 |
| AUC (pmol/ml min) | 0.30 | 0.149 | 0.008 | 0.34 | 0.159 | 0.005 |

AUC, area under the curve; BK, bradykinin; des-Arg$^9$-BK, des-Argin-ine$^9$-BK; SOLAR, Sequential Oligogenic Linkage Analysis Routines.

**Table 4 | Genotype distribution of the *XPNPEP2* C-2399A polymorphism in HSR+ and HSR− patients**

| | A and AA | CA | C and CC | Total |
|---|---|---|---|---|
| HSR+ | 4 | 2 | 7 | 13 |
| HSR− | 6 | 7 | 26 | 39 |
| Total | 10 | 9 | 33 | 52 |

HSR, hypersensitivity reaction.

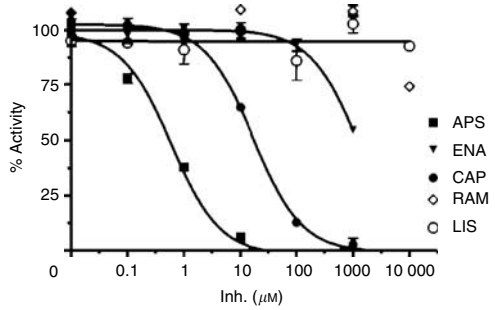


**Figure 4 | Specific and nonspecific inhibition of human recombinant soluble membrane APP.** Effect of increasing concentrations of various inhibitors on recombinant soluble hmAPP-catalyzed hydrolysis of K(Dnp)PPGK(Abz)$NH_2$ as described in Materials and Methods. The data were plotted to fit the one-site competition equation by nonlinear regression with GraphPad software and imposed constraints (−5.0 to 110% activities). $IC_{50}$ obtained were 0.6 ± 1.1 μM for apstatin (APS), 17 ± 1.1 μM for captopril (CAP), and over 1 mM for enalapril (ENA). Curves for lisinopril (LIS) and ramipril (RAM) did not converge (no inhibition).

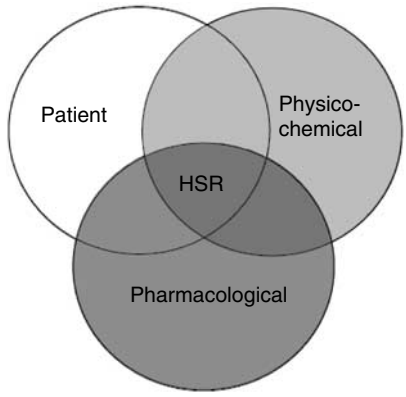


**Figure 5 | Hypothesis for a multifactorial nature of the HSR in hemodialysis.** HSR results from the meeting of at least three different factors: a physicochemical factor triggers the contact system of plasma and liberates BK, a pharmacological agent inhibits ACE activity, and a metabolic aspect which characterizes the capacity of the patient to metabolize kinins.

has also been found in human heart preparations where it contributes to BK metabolism.[26] It has been hypothesized that hmAPP on endothelial cells inactivates BK and locally produced des-Arg$^9$-BK. Suppression of hmAPP activity by apstatin, a commercially-available inhibitor, has highlighted the pathophysiological role of this metallopeptidase. However, the pharmacological effect of its blockade becomes evident mainly in the presence of an ACEi. In fact, inhibition of APP in rats synergizes the hypotensive action of injected BK, but this hypotension-potentiating influence is mostly seen in the presence of lisinopril.[27] In humans, apstatin has no effect on the inflammatory reaction induced by local subcutaneous BK injection in the forearm. However, the APP inhibitor acts synergistically with quinapril to augment the wheal response to exogenous BK.[28]

In this study, we measured APP activity in the plasma of HSR−, HSR +, and their relatives. For this purpose, we used the newly described, internally quenched fluorescent substrate K(Dnp)PPGK(Abz). This substrate, which mimics the $NH_2$-terminal sequence of the physiological substrate BK/des-Arg$^9$-BK, is highly specific for APP activity.[22]

With plasma samples obtained from the reference population,[12] we demonstrated that APP activity measured according this new analytical approach, was not only correlated with that published previously with the Arg-Pro-Pro (RPP) substrate ($r = 0.921$, $P < 0.001$), but also exhibited a higher correlation with the degradation rate of des-Arg$^9$-BK in the presence of an ACEi ($r = 0.822$, $P < 0.001$).[22] With this new substrate, we found significantly lower APP activity in HSR + compared to both HSR− dialyzed patients and the reference population.

As in our previous reports involving AE patients, the metabolic profiles of endogenous BK and des-Arg$^9$-BK in

HSR+ plasma were obtained by activating the contact system with glass beads in the presence of an ACEi. This mimics what happens *in vivo* when the plasma of an ACEi-treated patient comes in contact with a negatively charged membrane at the beginning of hemodialysis. Under our experimental conditions, we did not obtain evidence of any anomaly in the metabolism of the nonapeptide BK. Abnormal breakdown of des-Arg$^9$-BK, assessed by significant differences in the $\beta$ parameter that depended only on APP activity, led to a significant increase of maximal peptide concentration during the *in vitro* incubation period.

Similar metabolic anomalies among ACEi patients who developed HSR+ or AE indicate that one or several common genetic predisposition(s) may partially explain the pathophysiology of both adverse reactions. In the 14 families of HSR+ patients, we obtained heritability estimates for APP activity that were even higher than those calculated previously for AE.[21] This may be explained by different factors: the number of families (14 vs 8), the new analytical approach, but also the difference in the clinical context of side effects. We also found a significant association between low levels of APP activity (and the capacity to degrade des-Arg$^9$-BK) and the C-2399A SNP polymorphism in the *XPNPEP2* gene identified previously,[21] lending further credence to concepts that genetics, most commonly SNPs as candidate gene factors, determine interindividual variability in risk for the disease.[29] The C-2399A polymorphism was found to be predictive of low APP activity, but not sufficient for phenotype characterization, as is usually the case for quantitative traits.

As early as 1992, Hooper *et al.*[30] reported that several ACEi inhibit APP purified from the pig kidney. Given this, we could not exclude that an ACEi nonspecifically inhibited APP activity among HSR+ patients. This was particularly true in dialyzed patients with impaired renal excretion of most ACEi. We showed that, in addition to the selective APP inhibitor apstatin, the sulfhydryl-containing ACEi captopril could also inhibit APP at $\mu$M concentrations. Captopril was the first ACEi to be used clinically. It is characterized by a relatively weak inhibition constant ($K_i$ = 1700 vs 200 and 7 pM for enalapril and ramipril, respectively) and short half-life, necessitating multiple daily takes, at higher dosages, to achieve clinical efficacy. The concentration of captopril and other ACEi has not been documented in the plasma of dialyzed patients at the time of HSR episodes. Nevertheless, it is conceivable that, under these conditions, the high plasma concentrations needed to therapeutically inhibit ACE, coupled with poor clearance in dialyzed patients, could lead to the nonspecific inhibition of other metallopeptidases, such as APP, resulting in the accumulation of the $B_1$ agonist.

This nonspecific inhibition of APP by captopril and the important proportion of patients treated with the ACEi in the HSR+ group (8/14 vs 0/39 in HSR−) could plead for its role in the pathophysiology of some HSRs. Among the eight HSR+ patients treated by this ACEi at the time of the HSR+ episode, six had low or undetectable APP activity, but two (propositus of family X and XI) had activity (268 and 337 U, respectively) equal to or higher than the 50th percentile of the reference population. We could then hypothesize that APP activity could have been inhibited *in vivo* by captopril at the time of the HSR episode.

This study highlights the complexity of HSR, as is the case with AE and other side effects of metallopeptidase inhibitors (ACEis and vasopeptidase inhibitors). These observations also raise important questions about the regulation of APP enzymatic activity in human plasma, and, ultimately, about the pharmacological activity of $B_1$ agonists in humans, and particularly in dialyzed patients. The factors that regulate plasma APP activity are not known and must be identified. Plasma APP activity is regulated at least two different levels. First, at the level of synthesis in cells by yet unidentified transcriptional and possibly post-transcriptional mechanisms responsible for the quantity of APP bound at the cell surface by a glycosylphosphatidylinositol anchor. Studies are now ongoing in our laboratory, using cells expressing APP, such as endothelial cells and human embryonic kidney cells (HEK 293 cells), to better characterize these mechanisms. The second level at which plasma APP activity is controlled is at cleavage of the glycosylphosphatidylinositol anchor and release of the protein in plasma. Phospholipase C has been shown to be a candidate for such release. More interesting, however, is the recent report that ACE has glycosylphosphatidylinositolase activity.[31] As both APP and ACE are often expressed in the same cells, it will be interesting to see whether ACE levels affect membrane-bound APP.

Our study also raises a question about the pathophysiological meaning of our *in vitro* observations: the consequences *in vivo* of $B_1$ agonist accumulation during activation of the contact system of plasma in the presence of an ACEi. In a rat model, we have previously demonstrated a pro-inflammatory effect of des-Arg$^9$-BK when its corresponding $B_1$ receptor is expressed.[32] More recently, we have also observed in the pig that chronic enalapril treatment induces $B_1$ receptors in the kidneys.[33] However and contrarily to BK,[10,34] no des-Arg$^9$-BK concentrations have been reported until now in human and particularly in plasma of dialyzed patients. The present results could be used as a basis for a clinical study in which plasma BK and des-Arg$^9$-BK concentrations will be documented in HSR+ and HSR− patients. The association of these circulating levels of both $B_1$ and $B_2$ agonists with the clinical symptoms and the inflammatory status of these patients could elucidate the relative role of $B_1$ and $B_2$ receptors in these rare but potentially serious side effects. These results could also help us to understand the regulation of $B_1$ receptor expression in dialyzed patients and could objectivate the usefulness of non-peptidic $B_1$ antagonists, now in clinical development, for the treatment of metallopeptidase inhibitor side effects.[32,33]

## MATERIALS AND METHODS

### Participants

The study protocol was conducted according to French regulations and was approved by Ethics Committees from the Centre hospitalier de l'Université de Montréal (CHUM), the Institut de cardiologie de Montréal, McGill University, and the Université de Rennes (France). Informed consent was obtained from all participants. Patients were from five different nephrology-hemodialysis centers in France (Hôpital Universitaire and ARPDD, Reims, Centre Hospitalier La Beauchée, Saint-Brieuc, Centre Hospitalier Louis-Pasteur, Cherbourg, Centre Hospitalier Intercommunal de Cornouaille, Quimper) and one in Canada (Hôtel-Dieu de Saint Jérôme, Québec). Sampling took place at these sites between July 2001 and December 2003. Fifty-three patients on chronic hemodialysis, 39 HSR− and 14 HSR+, were enrolled in this study. Their main clinical characteristics are shown in Table 1. In addition, 171 relatives of the 14 HSR+ patients participated in a medical interview and donated blood samples for genetic linkage research (Figure 1).

### Hemodialysis

All patients were chronically hemodialyzed three times per week, for average session duration of 240 min. In all instances, the dialyzer, either flat sheet or hollow fiber, was equipped with the AN69 polyacrylonitrile membrane (Gambro-Hospal, Meyzieux, Lyon, France). The blood flow rate comprised between 250 and 350 ml/min. Unfractionated heparin was used as anticoagulant. The dialysate contained bicarbonate and was of the highest bacterial and endotoxin quality. Adequacy of dialysis was assessed by $K_t/V$ for urea higher than 1.2. Dialyzers were not reused. In those patients who developed HRS, ACEi was withdrawn, and they were shifted to another type of membrane, that is, a neutral membrane.

### Hypersensitivity reaction

The presence of HSR was defined on the basis of clinical criteria proposed recently by Bright *et al.*[35] in a survey of HSR 'as an incident involving two or more of the following symptoms occurring within 5–20 min of starting dialysis: abdominal cramps, nausea, vomiting or diarrhea; shortness of breath, chest tightness, wheezing, or bronchospasms; facial, labial and/or lingual swelling, AE or laryngeal edema; hypotension (>20 mmHg drop in systolic blood pressure); flushing, or warmth; numbness or tingling of the fingers, toes, lips, or tongue'. All the symptoms disappeared in 2–6 h after the end of dialysis, whether the session was completed or interrupted.

Dialyzed patients who presented HSR (HSR+ group) and their pedigree are represented in Figure 1. The etiology of renal failure was diabetes mellitus ($n=1$, propositus of family II); glomerular disease ($n=7$, propositus of family I, III, IV, V, VI, VIII, XIV); chronic interstitial nephritis ($n=2$, propositus of family VII, X); nephroangiosclerosis ($n=2$, propositus of family XI, XIII); polycystic renal disease ($n=1$, propositus of family IX); and unknown ($n=1$, propositus of family XII).

At the time of the HSR episode, eight patients were treated with captopril (propositus of family I, II, V, VI, IX, X, XI and XIV), four with enalapril (propositus of family VII, VIII, XII, XIII), one with benazepril (propositus of family V), and one with lisinopril (propositus of family III).

The group of patients who never presented any side effect while dialyzed in the same conditions as HSR+ served as the control group (HSR− patients). At the time of blood sampling, these patients were treated with the following ACEi: ramipril ($n=24$), enalapril ($n=5$), perindopril ($n=7$), and fozinopril ($n=3$).

### Laboratory investigations

***Blood and plasma samples.*** Venous blood was sampled at the start of dialysis and before heparin administration. Blood was collected in BD Vacutainers either on Na-citrate (1/10 v/v) for metabolic investigations or on $K_2$-ethylenediaminetetraacetic acid for DNA analysis. Citrated blood was centrifuged at 2000 *g*, plasma was collected and stored in sealed tubes at −80°C before shipping to the reference center. $K_2$-ethylenediaminetetraacetic acid samples were stored at room temperature until DNA extraction.

***Reagents.*** The ACEi enalapril was from Merck Frosst Canada (Kirkland, Québec, Canada); the ACEi ramipril was a generous gift from Dr W Linz (Sanofi Synthelabo-Aventis GmbH, Germany); BK and des-Arg$^9$-BK were acquired from Peninsula Laboratories (Belmont, CA, USA); the internally quenched fluorescent substrate K(Dnp)PPGK(Abz)[22] was synthetized by Professor A Carmona (Department of Biophysics, Escola Paulista de Medicina, UNIFESP, São Paulo, Brazil). All other fine chemicals and inhibitors were from Sigma (Montreal, Québec, Canada).

### Metabolic investigations

***Measurement of APP enzymatic activity in plasma.*** APP activity was assessed kinetically using the K(Dnp)PPGK(Abz) quenched fluorescent substrate. This original method was developed and validated recently in our lab.[22] The substrate allows sensitive kinetic measurement of plasma APP activity with intra- and inter-assay coefficient of variation (CV)% equal to 6 and 10%, respectively.[22] One unit of APP activity corresponds to 1 pmol of K(Dnp)PPGK(Abz) hydrolyzed per min/ml.

This analytical approach has the advantages of kinetic measurement of enzyme activity, and similar affinity of the enzyme for the new substrate ($K_m$: $20\pm5\,\mu$M) when compared to its natural substrate, des-Arg$^9$-BK ($K_m$: $56\pm13\,\mu$M);[22] it contrasts with values for the synthetic tripeptide Arg-Pro-Pro ($K_m$: $837\pm75\,\mu$M) previously employed to assess APP activity.

### Metabolism of endogenous BK and des-Arg$^9$-BK in plasma

***Contact system activation.*** Plasma was activated as described earlier for a group of normal healthy people[12] and AE patients.[19] Briefly, 1 ml of plasma was preincubated with enalapril for 20 min at 37°C in polypropylene tubes at a concentration (130 nM) which totally inhibited ACE activity. The contact system was then activated by incubation of the plasma with glass beads (37°C, with agitation). The reaction was stopped after various incubation periods (0–60 min) by adding cold anhydrous ethanol at a final concentration of 80% vol/vol. The samples were then incubated at 4°C for 1 h and centrifuged (4°C, 15 min, 3000 *g*) for the complete precipitation of protein. The supernatant was decanted and evaporated to dryness in a Speed Vac Concentrator (Savant, Farmingdale, NY, USA) before quantification of the immunoreactive peptides BK and des-Arg$^9$-BK.

***Measurement of immunoreactive BK and des-Arg$^9$-BK.*** The residues of evaporated ethanolic extracts were resuspended in 50 mM Tris/HCl buffer (pH 7.4) containing 100 mM NaCl and 0.05% Tween-20. After resuspension, residual BK and formed des-Arg$^9$-BK were quantified by two specific competitive enzyme immunoassays.[36,37]

Briefly, both immunoassays use purified polyclonal immunoglobulin G raised against the C-terminal portion of the peptides, responsible for the $B_1$ or the $B_2$ pharmacological activities.

Antibodies were raised in rabbits against the C-terminal part of BK covalently linked to bovine serum albumin using glutaraldehyde. The tracer used was BK labeled with digoxigenin-3-*O*-methylcarboxyl-ε-aminocaproic acid-*N*-succinimide ester (DIG-O-Su, Roche diagnostic, Laval, Québec, Canada) purified by high-pressure liquid chromatography and characterized by mass spectrometry and radioimmunoassay. Kallidin (Lys-BK) and BK are 100% crossreactive with these antibodies. None of the BK metabolites (des-Arg$^9$-BK or Lys-des-Arg$^9$-BK) present a significant cross-reactivity (<1%) with the anti-BK antibodies. The calibration curve was characterized by an $ED_{50}$ of 7.37 pg/well (50 μl). The CV for each value of the calibration curve is less than 5%. Intra- and inter-assay CV of BK measurements were lower than 4.0 and 7.4%.

For the quantification of des-Arg$^9$-BK, polyclonal antibodies were generated in rabbit against Cys-Lys-Aca-Lys-des-Arg$^9$-BK coupled with maleimide-activated keyhole Limpet Hemocyanin and purified by gel filtration (PD-10). The tracer was obtained by coupling des-Arg$^9$-BK to DIG-O-Su. After purification by hydrophobic chromatography, the tracer was also characterized by mass spectrometry and radioimmunoassay. Lys-des-Arg$^9$-BK and des-Arg$^9$-BK exhibit identical 100% immunoreactivity for the C-terminal peptide-specific antibodies. Neither BK nor Lys-BK crossreact in the assay and different metabolites of kinins present less than 0.1% cross reactivity. The calibration curve is characterized by an $ED_{50}$ of 6.96 pg/well (50 μl). The CV for each value of the calibration curve is less than 6%. The intra-assay CV calculated at three concentration levels varies between 5 and 8%, and the inter-assay CV is lower than 10%. The bound DIG-BK/DIG-des-Arg$^9$-BK is reacted with anti-DIG Fab labeled with horseradish peroxidase. The reaction was revealed by adding *o*-phenylenediamine and read by absorbance at 530 nm.

***Mathematical treatment of the kinetic profiles of BK and des-Arg$^9$-BK.*** The following mathematical model $y = k\ t^{\alpha}\ e^{-\beta t},\ t>0$ was fitted to the concentrations of endogenous BK and des-Arg$^9$-BK, measured at different times ($t$) for each plasma sample. This three-parameter ($k$, $\alpha$, and $\beta$; $k>0$, $\alpha$, and $\beta \geqslant 0$) model corresponds to a form similar to $\gamma$ distribution,[38] and has been described and validated earlier.[12] The $\alpha$ and $\beta$ parameters are respectively related to the shape of the first and the second part of the curve, corresponding to the formation and the degradation of each peptide. These $\alpha$ and $\beta$ parameters allow the calculation of other kinetic parameters: time of the maximum: the value of $t$ for which the maximum of the curve was obtained: $t = \alpha/\beta$; *maximum*: the value of the maximum of the curve which corresponds to the value of the curve for $t = \alpha/\beta$; AUC: the area under the curve which is mathematically given by $k\Gamma(\alpha+1)/\beta^{\alpha+1}$, where $\Gamma(\alpha+1)$ is the $\gamma$ function; half-life of formation ($t_f$): the value $t_f$ in the interval 0 to $\alpha/\beta$ for which $t^{\alpha}\ e^{-\beta t} = (0.5)\ (\alpha/\beta)^{\alpha}\ e^{-\alpha}$; half-life of degradation ($t_d$): the value $t_d$ in the interval $\alpha/\beta$ to $\infty$ for which $t^{\alpha}\ e^{-\beta t} = (0.5)\ (\alpha/\beta)^{\alpha}\ e^{-\alpha}$; slope of the half-life of formation: the value of the slope of the curve at half-life formation $= k e_f^{-\beta t_f} t_f^{\alpha-1}(\alpha - \beta t_f)$; and slope of the half-life of degradation: the value of the slope of the curve at half-life degradation: $k e_d^{-\beta t_d} t_d^{\alpha-1}(\alpha - \beta t_d)$.

## Genetic investigations

***Heritability.*** To test the hypothesis that genetic factors could influence the variability of APP activity and of kinetic parameter values for the metabolism of BK and des-Arg$^9$-BK, heritability was estimated among the combined families of the 14 HSR+ patients. For this analysis, probands were not excluded on the basis that the pedigrees were not selected for APP activity levels and covariates were not included in the analysis.

***Genotyping.*** A commercially available protocol (Gentra, Minneapolis, MN, USA) was applied for DNA extraction. Genotyping of C-2399A SNP, described elsewhere,[21] required the modification of an allele-specific polymerase chain reaction assay.[39]

The SNP genotypes served two purposes: (1) to compare allele and genotype frequencies between HSR+ and HSR− patients; (2) to evaluate associations between SNP genotypes and phenotypes (APP activity or different kinetic parameters of BK and des-Arg$^9$-BK) in both groups (HSR− and HSR+).

## Pharmacological investigations

***Production and purification of human recombinant soluble membrane APP.*** A secreted form of wild-type human APP was engineered and fully characterized.[22]

***Inhibition studies of recombinant APP with metallopeptidase inhibitors.*** Kinetics parameters were measured with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The data were plotted to fit the one-site competition equation by nonlinear regression, with imposed constraints (−5.0 to 110% activities).

All enzyme activity assays were performed at 37°C. Assay mixtures minus recombinant APP served as controls. Stock solutions of different inhibitors (ramipril, apstatin, enalapril, captopril, and lisinopril) were successively diluted in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid 0.1 M (pH 7.4). The final concentrations tested ranged from 0 to 1 or 10 mM. Reactions were in triplicate and incubated at 37°C for 90 min, with or without a metallopeptidase inhibitor. Hydrolysis of the fluorogenic substrate K(Dnp)PPGK(Abz) was performed as follows: reactions were in 150 μl volume in opti 96-well plates containing 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (pH 7.4), 10 μM substrate, and purified recombinant enzymes. Fluorescence was measured kinetically (every 5 min) with a FL600 microplate fluorescence plate reader (BioTek, Winooski, VT, USA) with excitation of 340 nm and an emission of 420 nm.

***Statistical analysis.*** For kinetic parameters and enzyme activities, the means of the parameters for the two groups (HSR+ and HSR−) were compared by *t*-test with the Satterthwaite-Welch approach, taking into account the possible heterogeneity of variances[40] using SPSS, version 13.0. $P<0.05$ values were considered statistically significant.

The heritability coefficients of APP and the different kinetic parameters of kinins were calculated on *z*-scored values by SOLAR (Sequential Oligogenic Linkage Analysis Routines, version 2.1.4 Official)[41] with robust estimation of means and variance according to *t*-distribution. $P<0.05$ values were considered statistically significant.

The APP means were compared between the genotype groups by one-way analysis of variance, followed by linear contrasts; a *t*-test was used for the recessive genetic model.[40] SNP association results were statistically significant at $P<0.001$.

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